THE NC1 DOMAIN OF TYPE X COLLAGEN: A NOVEL NUCLEATION SITE FOR TRIMER AND MULTIMER FORMATION REVEALED BY MOLECULAR ENGINEERING

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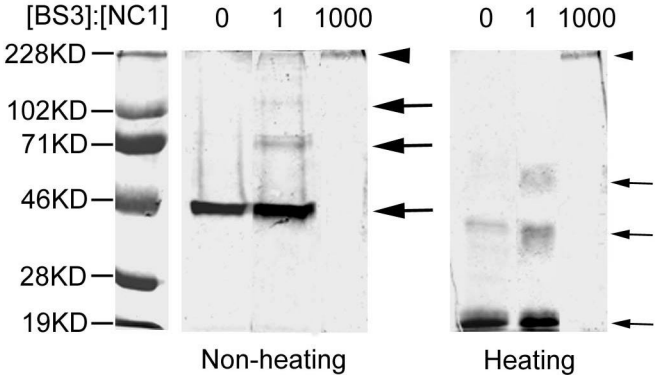
Introduction: Type X collagen is expressed specifically by hypertrophic chondrocytes at the transition of cartilage to bone during endochondral bone formation. Mutations in the human collagen α1(X) gene result in Schmid metaphyseal chondrodysplasia (SMCD). Type X collagen consists of three α1(X) chains. Each α1(X) chain contains three domains, a central helical domain (Col 1) flanked by the C-terminal and N-terminal non-collagenous domains (NC1 and NC2). Interestingly, most of the mutations in the SMCD occur in the C-terminal NC1, which is highly conserved among species. The functions of NC1, however, remain unclear. It was proposed that NC1 is important for the triple helical assembly of the collagen. However, a homologous NC1 domain has been found in the C-terminus of non-collagenous molecules such as multimerin and precerebellin. The aim of this study is to test the hypothesis that NC1 is sufficient to direct trimer and multimer formation, even in a non-collagenous molecule. We have expressed and characterized two recombinant peptides containing the NC1 domain from avian α1(X). One peptide contains only the NC1 domain, and the other peptide contains a non-collagenous A domain at the N-terminus and the NC1 domain at the C-terminus. Both peptides form trimers and a series of multimers via NC1. Therefore, the NC1 domain of collagen X is a novel nucleation site for trimer and multimer formation, for both collagenous and non-collagenous molecules.

Method: For the NC1 peptide, a cDNA fragment correspondent to the 162 amino acid sequence of the NC1 domain from avian α1(X) was amplified by PCR. The fragment was then cloned into a pQE32 (type IV) (Qiagen, Santa Clarita, CA) to carry a six histidine-tag at its N-terminus. For the A-NC1 construct, a cDNA fragment (582 bp) of the A2 domain from avian CMP was amplified from a mini-CMP cDNA[1] by PCR and then ligated into the XmaI site 5' upstream of the NC1 insert in the expression vector. This ligation created a chimeric molecule with the A2 domain at its 5' end and the NC1 domain at its 3' end. The recombinant plasmids were transfected into competent M15 *E. coli*, and expression of peptides were induced with 2 mM IPTG. The peptide was purified either under native condition on a Ni-NTA slurry column (Qiagen), or under denatured in urea buffer on a Ni-NTA slurry column according to the manufacturer's suggestion. Then the slurry was divided into two parts for refolding in liquid and solid phases. For the liquid phase folding, the denatured peptide was eluted with elution buffer, followed by dialysis in urea step gradient buffers (6-0 M Urea, 500 mM NaCl, 20% glycerol, 20 mM Tris-Cl, 1 mM PSMG, pH 7.4) in an 8 hour refolding process. For the solid phase folding, the peptide was first refolded on the column with the same urea step gradient buffers over the same period of time. After refolding the peptide was eluted with imidazole elution buffer. The A2-NC1 chimeric peptide was purified and refolded on a solid phase, identical to the denatured procedure described above. Crosslinking was carried out with BS^3 (Pierce, Rockford, IL). Ten μM of the refolded NC1 peptides were mixed with various concentration of BS^3 and incubated at room temperature with gentle shaking for 1 hour. The samples were loaded on a 10% SDS-PAGE for electrophoresis.

Result: To determine whether NC1 forms a trimer, the recombinant NC1 peptide was expressed in E.coli, and purified under both native and denatured conditions. The native NC1 peptide existed as a 45KD trimer on a SDS-PAGE gel. This is the same molecular weight as the NC1 trimer purified from type X collagen in hypertrophic cartilage. This suggests that the native NC1 peptide alone is sufficient to form a trimer. The denatured NC1 peptide existed as a 20KD monomer and a 40KD dimer. After refolding in vitro, it also formed a 45KD trimer. This suggests that NC1 initiates trimer formation. This trimeric association is extremely stable. It is resistant to denaturing conditions such as SDS detergent and reducing conditions, and it is highly thermal stable. The trimers were resistant to thermal denaturation up to 90°C under both reducing and non-reducing conditions. This indicates that disulfide bonds are not involved in the stability of the trimeric association. To test whether the NC1 trimers form higher-order multimers, we performed a crosslinking experiment with a crosslinker BS^3 (Bis(sulfosuccinimidyl) suberate) that can crosslink neighboring peptides whose distance is no more than 11.4 Å. The NC1 peptides were incubated with BS^3, in a series of solutions with increased molar ratios of BS^3 to NC1 (Figure). In a solution with equal concentrations of BS^3 and NC1, a ladder of bands appeared (Figure, arrows in the middle panel). The smallest of this series of bands was the 45 KD NC1 trimer. Therefore, the NC1 trimer can form higher-order multimers. After heating at 100°C, the crosslinked sample included at least a



20 KD monomer, a 40 KD dimer, and a 60KD trimer (Figure, arrows in the right panel). The unheated native NC1 trimer migrated at the 45 KD position, faster than a heated denatured trimer. This is due to the compact configuration of the native peptides. At a condition with excessive crosslinkers (1000 times of the [NC1]), the NC1 peptides became a high molecular polymer under both heating and non-heating conditions (Figure, arrowheads). To test whether the NC1 domain facilitates trimer formation in a non-collagenous molecule, the chimeric A-NC1 peptide was expressed in E.coli and purified under denatured conditions. The refolded peptide contained three forms on a SDS-PAGE: a 40 KD monomer, a 100 KD trimer, and a 200 KD hexamer. After heating at 100°C, both of the multimeric forms were shifted to the 40 KD monomeric form. Thus, the A-NC1 is capable of forming multimers via the NC1 domain.

Discussion: We have demonstrated here that the peptides containing the NC1 domain are sufficient to form trimers and higher-order multimers without any triple helical residues. This suggests that the NC1 domain alone is sufficient for the proper chain association and trimeric assembly of type X collagen during synthesis. Our data suggests that NC1 contributes to the extraordinary stability of type X collagen. It is conceivable that mutations in NC1, such as those in the SMCD, may affect trimer and multimer formation of the molecule, or the stability of the multimeric association. With molecular engineering, we have shown for the first time that the NC1(X) domain is capable of directing trimer and multimer formation in a non-collagenous molecule. This suggests that the NC1 domain is a novel nucleation site for trimer and multimer formation, even in a non-collagenous molecule.

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